6.1 Polymerase Chain Reaction (PCR) Introduction

The polymerase chain reaction technique employs oligonucleotide primers to amplify segments of genes specific for the target pathogen. Reverse transcriptase-PCR (RT-PCR) employs an initial reverse transcription step so that complimentary DNA can be amplified from viral RNA. DNA or RNA is extracted from various fish tissues and laboratory assay products, such as cell culture supernatant containing viral agents, and amplified using forward and reverse primer sets. In some instances for either method, the initial amplified product may be re-amplified using an additional "nested PCR" technique. The DNA products are then visualized by gel electrophoresis.

Specific details for sample preparation, DNA/RNA extraction, primers and cycle conditions appear in each chapter under the specific pathogens. However, certain PCR protocols and precautions are pertinent to all assays and these are described in this chapter. The PCR quality assurance and control procedures outlined below are extremely important when performing all PCR assays (Ennis et al. 1990).

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service, the United States government, and/or the American Fisheries Society. Any comparable instrument, laboratory supply, or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

6.2 PCR – Quality Assurance/Quality Control

A. General Considerations

1. Quality control is critical to all steps of the PCR process, beginning with collection of samples in the field. It is important that the person performing sample collection use the precautions outlined in Chapter 2 Sampling to avoid cross-contamination.

2. Work surfaces should be decontaminated by washing with 10% chlorine (or commercial reagents like "DNA Away") to hydrolyze possible DNA contaminants. All sample racks and reusable equipment should be washed in DNA-away and autoclaved after use. Spray/wipe pipettors and working areas with DNA or RNAase -Away and turn UV on for at least 30 minutes after use (UV light damages DNA).

3. **Wear and change gloves often**. This helps prevent spread of amplified DNA or contamination of sample DNA with nucleases naturally occurring on the skin that will degrade the sample DNA. Always change to a fresh pair when leaving and entering PCR reagent mixing areas. Change gloves whenever contamination between samples is possible.

4. Employ aerosol resistant pipette tips and/or positive displacement pipettors during all extraction and amplification procedures. Separate pipettors should be dedicated for use with reagents only and another set for use with amplified products only.

5. Mix and aliquot pre-amplification ingredients under bench top UV cabinet and NEVER contaminate this area with sample material or amplified DNA product.

6. **One aerosol drop of PCR product may contain thousands of strands of DNA, which can easily contaminate reagents!** Therefore, three separate areas of lab space are necessary to reduce the risk of contamination.

    a. Master Mix (MM) Area with UV Hood
    For mixing and aliquotting master mix reagents. Supply area with dedicated pipetters, ideally positive displacement pipettor/tips. **No samples or amplified DNA is to be handled in or near this area!**

    b. Sample Loading Area with UV Hood and Dedicated Pipettor
    For loading of extracted (template) DNA from samples**.**

    c. Amplified DNA Area
    Supplied with pipetter dedicated for **amplified PCR product ONLY.** Handle any amplified PCR products in this area only, and clean area and equipment thoroughly with "DNA Away" type solutions after working with amplified DNA.

7. Provide separate storage areas for RNA and DNA samples, amplified DNA, and PCR reagents.

8. Controls

    a. Extraction controls
    A known positive tissue sample (or tissue spiked with target pathogen DNA) and a known negative tissue should be processed with the test samples to ensure that the DNA extraction was successful and contamination did not occur.

    b. PCR Controls
    Sterile water (negative) and the known positive DNA and negative controls from previous extraction (positive) will ensure that the PCR process was successful and that contamination did not occur.

9. Primers
    Newly received primer batches should first be tested on known positive/negative controls.

10. Dispose of trash containing amplified DNA products frequently.

B. Sample Processing

1. Tissue samples should be collected on a clean bench top, which has been disinfected using a 10% chlorine (or "DNA Away") solution if possible. If collected in the field, use a disposable work surface between each lot of tissue collected (paper towel, foil etc.)

2. Use sterile collection utensils between each lot of fish tissue collected. If data from individuals is of concern, use separate utensils for each individual. **Alcohol will not effectively decontaminate DNA from utensils.** If individual utensils are not available, flaming metal utensils between samples will effectively remove contaminants from previous samples.

3. Keep samples cold and freeze as soon as possible at or below -20^{o}C until processing can be accomplished.

4. RNA is extremely sensitive to enzymes present in sample tissues. Samples collected for RT-PCR should be frozen immediately and transported on dry ice. An RNA stabilizing buffer can also be used and does not require that samples be frozen immediately.

C. Extraction of DNA or RNA from Samples

Individual protocols will vary in specific steps for extraction of genetic material; however, the following general considerations should be employed:

1. Use micro centrifuge tubes with locking or screw-cap lids. Heating of extraction solutions causes unlocked caps to pop open, releasing aerosols that can cause cross-contamination between samples and controls. Pulse spin in the microcentrifuge before opening DNA sample tubes so that the lids are dry before opening them. This will help in preventing cross-contamination.

2. Use the accurate amount of tissue suggested by the extraction kit manufacturers. If this is exceeded, proper lysis of tissues will not be accomplished.

3. Always run positive control samples as well as negative (water and negative tissue samples) from the start of the extraction process, through amplification to electrophoresis. These controls will allow for detection of contamination as well as assure that the extraction was successful. This is the only means of assuring validity of the assay and its results.

4. **Quantification of DNA**
   if the protocol used advises that extracted products be measured using a spectrophotometer to ensure that enough DNA or RNA was successfully extracted, refer to quantification guidelines in 6.5 Analysis of Extracted DNA using an UV Spectrophotometer.

D. Interpretation of PCR Results

Use of the appropriate controls should allow you to assess the integrity of your PCR result.

1. False-negative reactions may result from insufficient DNA extraction, excessive amounts of DNA, PCR inhibition, improper optimization of the PCR, or human error (e.g. loading errors).

2. False-positive reactions may result from contamination either directly from the sample lot being tested or from previously amplified target DNA.

3. For further help in troubleshooting, see PCR Protocols: A Guide to Methods and Applications (Ennis et al. 1990).

6.3 PCR Protocols

A. Preparation of Amplification Reaction Mixture

Specific amplification protocols may require one or two amplification reactions.

Note: samples and reagents should be kept cold either on ice or in a frozen cryo-rack during all assembly procedures.

1. Using 3.A3.A Worksheet A – PCR Sample Data/Log Sheet or 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.

2. Under a UV cabinet, prepare "Master Mix" (MM) using pathogen-specific protocols in the worksheets in each chapter. Calculate the amount of each reagent to go into the MM according to the number of samples to be processed. Add PCR reagents, **except for sample DNA,** in the order listed on the worksheet, adding water first and Taq polymerase last. Keep all reagents cold in frozen cryo-rack or on ice during mixing and return them to freezer immediately after use.

   Note: Prepare enough MM for three more samples than actually being tested to compensate for retention of solution in pipette tips and tube.

3. Place specified volume of MM into each PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

4. In the sample preparation area, load specified volume of each sample DNA to the appropriately labeled PCR tubes. To avoid cross contamination, always change tips between samples and avoid touching the sides of the tube. Close caps tightly.

B. Running the PCR

All general considerations should be employed including the following:

1. Load the sample tubes into the wells (follow manufacturer's recommendations).

2. Program thermocycler for appropriate cycle conditions and run reaction.

3. Before loading into thermocycler, give tubes a "quick-spin" to ensure that all reagents and sample are drawn down from sides of tube.

4. Thermocycler should be programmed for the specific PCR condition used for each pathogen (details under pathogen).

5. After cycling, tubes may have a ring of condensation near rim of cap. Before opening tubes, perform a "quick-spin" to draw this fluid down into the reaction area of the tube and reduce the

possibility of aerosol contamination upon opening tubes.

6. PCR products can be refrigerated for up to a month following amplification (or for longer storage they may be frozen at -20˚C).

C. Detection of Product

Procedures for preparing the gel (refer to specific manufacturer's guidelines for preparation of gels and electrophoresis chambers):

1. Assemble the gel tray and position well comb in the tray according to manufacturer's recommendations.

2. Prepare 1 X TAE (6.4 Reagents) buffer with distilled water to volume adequate for gel and running buffer.

3. Prepare 1.5 to 2% agarose gel according to the volume recommended for specific gel forms used.

    a. Weigh appropriate amount of agarose and add to proper volume 1X TAE buffer.

    b. Heat solution to near boiling until agarose is completely dissolved.

    c. Allow solution to cool to about 65^{o}C, then pour agarose solution into gel tray. Avoid the formation of bubbles.

    d. Allow gel to cool completely for about 30 minutes and then carefully remove the comb.

    e. Position the gel into the chamber with the sample end (wells) positioned closest to the negative (black) electrode.

    f. Slowly fill the chamber with the remaining 1 X TAE buffer solution until the top of the gel surface is submerged.

4. Load samples into wells as indicated for each assay

    a. For each tube of PCR product to be visualized, mix 2 µL of gel loading dye (6.4 Reagents) to every 10 µL of PCR product needed to fill each well formed in the gel. Mix the sample and the dye by repeated expulsion prior to loading.

    b. When the sample and the dye are adequately mixed, carefully place the pipette tip containing the mixture over an individual well of the agarose gel, and load the well with the sample. Repeat this procedure for all the wells, being sure to include the DNA molecular weight standard (one with bands at 100 bp increments in the 100 to 1,000 bp range) for base pair (bp) reference and positive and negative controls

    c. Document gel lane assignments for each sample and control on 3.A3.B Worksheet B – Amplification of Nucleic Acid by PCR or 4.A1.B Worksheet B – Amplification of Nucleic Acid by PCR and allow for at least one lane for a DNA ladder reference.

D. Electrophoresis

Approximately 80 volts for 35 minutes or until tracking dye front approaches the edge of the gel (this is dependent on gel width, so refer to manufacturer's recommendations).

E. Staining the Gel

Remove gel and tray and place in ethidium bromide (EtBr) solution (6.4 Reagents) for 15 to 20 minutes.

Note: EtBr solution can be reused and stored in a dark plastic tray container with a secure lid. **EtBr is very toxic** and binds with all DNA (including yours); follow appropriate manufacturer warnings! For safe proper disposal of expired EthBr solutions see Sambrook et al. or check with your local biotech supply retailer for specific products designed to remove EthBr from solution for disposal.

F. De-Staining the Gel

In water for 5 to 60 minutes. De-stain water should be handled and disposed of appropriately (see 6.3.E "Staining the Gel").

G. Visualize the DNA

1. Place gel on a UV light source and carefully record locations of bands on positive control samples in relation to the DNA molecular weight standard. Band locations of positive controls should be at anticipated locations according to primers used.

   Note: use UV protective goggles or face shield.

2. Note any unusual band occurrences. Negative controls should not have any bands. Contamination suspicions indicate the samples should be re-run from template DNA tube.

3. Photo document all PCR gels and attach to 3.A3.G Worksheet G – Photodocumentation of PCR Product Gel or 4.A1.C Worksheet C – Photodocumentation of PCR Product Gel (or provide reference for finding the photo documentation).

6.4 Reagents

REAGENT:	REFERENCE:	DIRECTIONS TO PREPARE:
TAE BUFFER 10X	Sigma T-4035	Comes in prepared packets, add DI water and qs to 1L . Label as 10X- STOCK (store at RT). Also can prepare a 50X stock (see Sambrook et al.).
TAE BUFFER 1X WORKING SOLUTION	TAE-1X	Diluted 1:10 from 10X stock. Label as TAE - 1X (store at RT).
PCR LOADING BUFFER -OR- LOADING DYE	Sigma P-7206 LOADING DYE 6X	Pre-made 6X concentrate, ready to use (store -20˚C). Prepare in-house (per Sambrook et al. (store at 4˚C): Bromophenol blue 0.25%, Xylene cyanol 0.25%, Glycerol 30.0 %

ETHIDIUM BROMIDE - Recommend buying EtBr already in solution to minimize working with this hazardous compound, or it can be prepared as follows:

Note: USE CAUTION IN PREPARING EtBr SOLUTIONS: Follow all MSDS precautions. Wear gloves, avoid all contact with skin, eyes, and respiratory system. LABEL ALL BOTTLES WITH "CHEMICAL CARGINOGEN."

EtBr STOCK SOLUTION - 10mg/mL	Ethidium Bromide 100 mg DI water 10 mL Label as EtBr STOCK (10mg/mL). Protect from light (store at RT).
EtBr-WORKING SOLTION - 4.0ug/mL* WORKING STAIN SOLUTION	Add 200 µL STOCK SOLUTION to 500mL water Label EtBr - Working Solution (store at RT).

*References may suggest weaker working solutions (0.5 µg/mL) and staining periods of 45 to 60 minutes.

6.5 Analysis of Extracted DNA Using an UV Spectrophotometer

DNA quantification is achieved routinely with the use of spectrophotometry. The 230, 260, and 280 wavelengths provide the readings for quantification and purity and the 320 wavelength provides a reading for background compensation.

Standard quantitative conversion factors for nucleic acids are as follows:

1 absorbency unit at 260 nm of ds DNA = 50 ng/μL
1 absorbency unit at 260 nm of ss DNA = 33 ng/μL
1 absorbency unit at 260 nm of ss RNA = 40 ng/μL

Absorbance values for the 260 nm readings need to be in the linear range (between 0.1 and 1) for quantitation to be valid. If the absorbance at 260 nm (A260) is above 1.0 the sample needs to be diluted. If the sample is below 0.1 there is negligible DNA in the sample.

Relative purity of the DNA sample can be determined by the 260/A280 ratio. If the sample is pure nucleic acid, the ratio should be approximately 1.9. Excess protein in the sample will raise the absorbance at 280 nm thereby reducing the A260/A280 ratio.

One inexpensive instrument for this purpose is the GeneQuant II (Pharmacia Biotech) although other makes and models are available. The GeneQuant II is a spectrophotometer specific for obtaining concentrations of either double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), or RNA in units of weight, molar fraction, moles of phosphate, and total molecules. The instrument is capable of measuring the RNA or DNA using UV wavelengths at 230nm, 260 nm, 280 nm, and 320 nm simultaneously. If a standard spectrophotometer is all that is available, then conversion to pmoles /μL (i.e. uM) from ng/μL can be accomplished with the following table.

Amount of primer (ng) needed to equal 10 pmol:

Primer Length	ng of Primer Equal to 10 pmol
15	50
16	53
17	56
18	59
19	63
20	66
23	78
24	80

6.6 References

Ennis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White, editors. 1990. PCR protocols: a guide to methods and applications. Academic Press, Inc.

Sambrook J., E. F. Fritsch, and T. Maniatis. 1987. Molecular cloning: a laboratory manual. 2nd Edition. Cold Springs Harbor Laboratory Press. Plainview, New York.